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A need for rapid characterization of bacteria for prompt clinical diagnosis and early warning of unusual bacteria in the atmosphere has prompted a number of different approaches to attempt to circumvent the usual time-consuming tedious procedures commonly employed. None appear to have been very successful.

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20. Abstract Continued

In the present case, state-of-the-art scanning electron microscopy (SEM), energy dispersive x-ray analysis (EDXA) and automated image analysis by computer were employed in a survey of thirteen species of bacteria.

The organisms were first cultured and cloned to check for mutants or other contaminants and were then fixed, stained and dispersed, free of debris on a structure-free background. Next, the magnification and other SEM operating conditions were controlled, and the binary image in the secondary electron mode was made to correspond to the grey scale image. Finally, the computer print out of each individual organism was checked with the binary image to pick up inconsistencies in the data arising from the characteristics of the stereologic based program.

The computer data for the mean width and average length were plotted on a ternary diagram. After the protocol was established and the variables controlled in each sample, about 100 organisms were measured and then the entire procedure was duplicated to determine reproducibility. In three runs on thirteen organisms, the reproducibility was generally around 1%, easily sufficient to characterize each organism.

Subsequent studies of the entire series would very likely permit rapid characterization of these as well.

This preliminary study indicates a feasibility that the enterics and other bacteria of interest to man could be rapidly identified by their genetically characteristic morphology.

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INTRODUCTION

The genetic mechanisms of bacteria which determine size and shape are the means to a deeper understanding of the basis of cellular morphology. As characters for classification of organisms, size and shape, if constant under known conditions for a specific type, give values which are limited only by the precision and reproducibility of measurement. An attempt to characterize bacteria by using a Coulter counter was reported[1], but we know of no other effort similar to the following study. The enteric bacteria were selected for a morphometric study because of the extent of knowledge of their characters and because, as individual cells, they are suitable subjects for a particulate analysis program.

At first, ten samples were obtained from the American Type Culture Collection (ATCC) which they cultured from lyopholyzed material and presented to us on Nuclepore filters. The organisms appeared clumped in the SEM, however, it was possible to take measurements from micrographs at 10,000X. This established statistically significant differences in width and length.

The preparation of similar samples which could be automatically measured by image analysis computer and then the refinement of the procedure to produce precise reproducible data are the subject of this communication.

MATERIALS AND METHODS

Bacterial Preparation

Lyopholyzed cultures of various enteric bacteria purchased from ATCC were reconstituted in nutrient broth (England Laboratories, Beltsville, Md.,). Biochemical analysis of these microorganisms confirmed the identity of the ATCC strains. Nutrient agar and nutrient broth were used for all species in order to maintain consistency. Nutrient agar yielded abundant growth in less time than the nutrient broth. Agar plates were cultured with the specific bacteria using a standard plate dilution technique. For growth consistency a cloned colony from the isolation plate was subcultured to another nutrient agar plate. After 18 hours incubation at 37°C, 6cc of 2.5% gluteraldehyde (in PO_4 buffer) was poured onto the colonies for suspending and fixing the bacteria. Colonies were mixed with the fixative and policed into a conical 15ml test tube. The tube was vortexed 1-2 minutes to suspend clumped microorganisms. Organisms were refrigerated at 6°C for 24 hours which allowed heavier particles to settle while the upper supernatant levels contained well dispersed singular rods. The upper supernatant contained 1500 million organisms per ml measured by nephelometric densitometry.

Bacterial cells were maintained in as natural a state as possible, thus avoiding any chemical or traumatic effects such as osmification or centrifugation. Preliminary experiments showed that osmium caused minor clumping. Slow centrifugation (500 - 600 rpm) did not eliminate

minute clumps. Faster centrifugation (1000 - 3000 rpm) caused bacteria to clump in doubles and triples. The gravitational method yielded dispersed singular organisms in the upper supernatant level (Figure 1).

One drop from the upper supernate level was placed onto a non-porous Nuclepore membrane which was attached to an SEM stub. Singular bacteria adhered to the Nuclepore, thus allowing the specimen to be washed and stained with 5% uranyl acetate and 0.4% lead citrate.

Image Analysis

The analytical system used[2] for characterizing bacteria according to size and shape consists of an SEM (Hitachi HHS-2R) and a mini-computer (LeMont Interdata, Model 7/16) to direct the electron probe. With this system the electron probe is rastered in a digital array on a dispersion of bacteria. When a bacterium is encountered, an increase in the secondary electron signal is detected. These increased signal points are called "on" points, and by successive, computer-controlled perpendicular bisections of "on" point lines within the bacterium (starting with the first raster line to intersect the bacterium), the center of the bacterium is defined. Through this point, the beam makes eight, equi-angled transects, the largest chord defining the length and the smallest chord defining the width. These parameters are tabulated, and the rastering process resumes. The X and Y coordinates of each organism are indicated permitting a visual recheck of the image analysis program. Center points within the bacterium are also recorded so that subsequent encounters with the same organism will be ignored.

When the analysis is complete, a summary chart is provided with average area, average length, average width and average length-to-width ratio. The computer is programmed to eliminate oversized artifacts and therefore it measures all singular bacteria within the field. A magnification of 2000X was selected to allow the largest number of organisms in a field to be counted which would at the same time provide adequate resolution of dimensions. Off and on particle picture point densities were chosen which provided reasonable analysis speed and resolution for the magnification selected. These were 512 and 819, respectively. A working distance of 15mm was selected to minimize the shadow effect of the lower signal probability from surfaces opposite to the secondary electron detector. Specimen height was carefully controlled at 0° tilt. To get the secondary image to correspond closely to the binary image, a high contrast signal was obtained so that the peaks due to the organism had steep sides approaching the vertical axis. (Figures 2a and 2b).

RESULTS

After the protocol was established and the variables controlled in each sample, approximately 75 - 100 bacteria were measured. The procedure was repeated several times to determine reproducibility. In three separate runs of the thirteen microorganisms investigated, reproducibility was generally around 1%, easily sufficient to characterize

each organism. Computer for the mean width and average length were plotted on a ternary diagram (Figure 3).

Functions of length, width and length plus width of the organisms are plotted from three axes as % of the range which runs along the perpendicular bisection to the opposite sides as a ternary diagram. Three runs were combined so that the reproducibility could be demonstrated. Each point of the illustrated triangles represents one separate run from culturing to analysis. For some organisms the runs match so closely that triangles were barely discernible and three points were plotted on a short straight line.

The most consistent data in the secondary electron mode was provided by air dried cells flattened against the substrate. A fault of the stereologic image analysis program was found to produce occasional too short width measurements. These outliers were detected by inspection of the print-out data for each organism, eliminated from the data and the mean width recalculated. In the same manner, organisms occasionally found side by side were eliminated.

DISCUSSION

The range of measured dimension variation of enteric organisms cultured under specific conditions is narrower than expected. It is easily accounted for by what we would expect from the equipment and the method as developed so far. This raises the prospect that with improved equipment and procedures we may further define the limits of dimensions with the prospect of exploring the characterization of

microorganisms of diverse origins beyond the species level. Genetic variations in colony morphology [3,4,5] of bacterial strains may well reflect slight morphologic differences in the individual cells which improved methodology could discriminate.

It is within the art of current computer technology to translate data for an organism such as is presented here directly into a definitive or probable identification. A substantial improvement in image dimensional fidelity would be anticipated from the use of a backscattered electron detector and critical point dried cells.

The x-ray peaks representing the U and Pb stains were almost 1:1 for all three organisms. While this did not provide additional criteria to distinguish the organisms from each other, it could well distinguish bacteria from non-bacterial contamination as in the direct examination of non-cultured material. The computer analysis system which was employed in this study is programmed to distinguish each particle by its chemical composition. All non-bacterial material above and below the set dimension limits and not containing a similar ratio of U to Pb can be eliminated by the program.

Application to virus characterization is conceivable in that the resolution of commercial SEM's is better than 10 nm. The U and Pb staining can distinguish DNA from RNA, respectively, and under controlled conditions this could be quantitated and compared to reference data. The system employed in this study has been applied to the detection and counting of virus on cell culture surfaces.[6]

The particle analysis program employed in this study may be improved by a fast Fourier transform (FFT) program which emphasizes repeating spatial frequencies. This could improve the effective resolution and increase application to complex organisms such as diplococci, streptococci, sarcini, etc.

While the precision and reproducibility of the data so far seems promising, results are confined to 13 organisms representing 11 genera and three species of one genus (Enterobacter). There are at least 26 more species of the common enterics and a great many strains of each. These must be analyzed and the data recorded before the value of this approach to microbiology can be fully appreciated. The effect of growth conditions, including adaptation to culture media, is yet to be explored.

In spite of these uncertainties, microbial morphometry under the conditions described can be used to study the genetic basis for the determination of size and shape as well as to characterize bacteria.

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Acknowledgement:

We wish to acknowledge the technical assistance of Ms. Sharon Moore for her contribution to the preliminary research effort.

Fig. 1. Well dispersed Salmonella enteriditis microorganism adhered to a polycarbonate membrane (nuclepore). Bar length = 1um

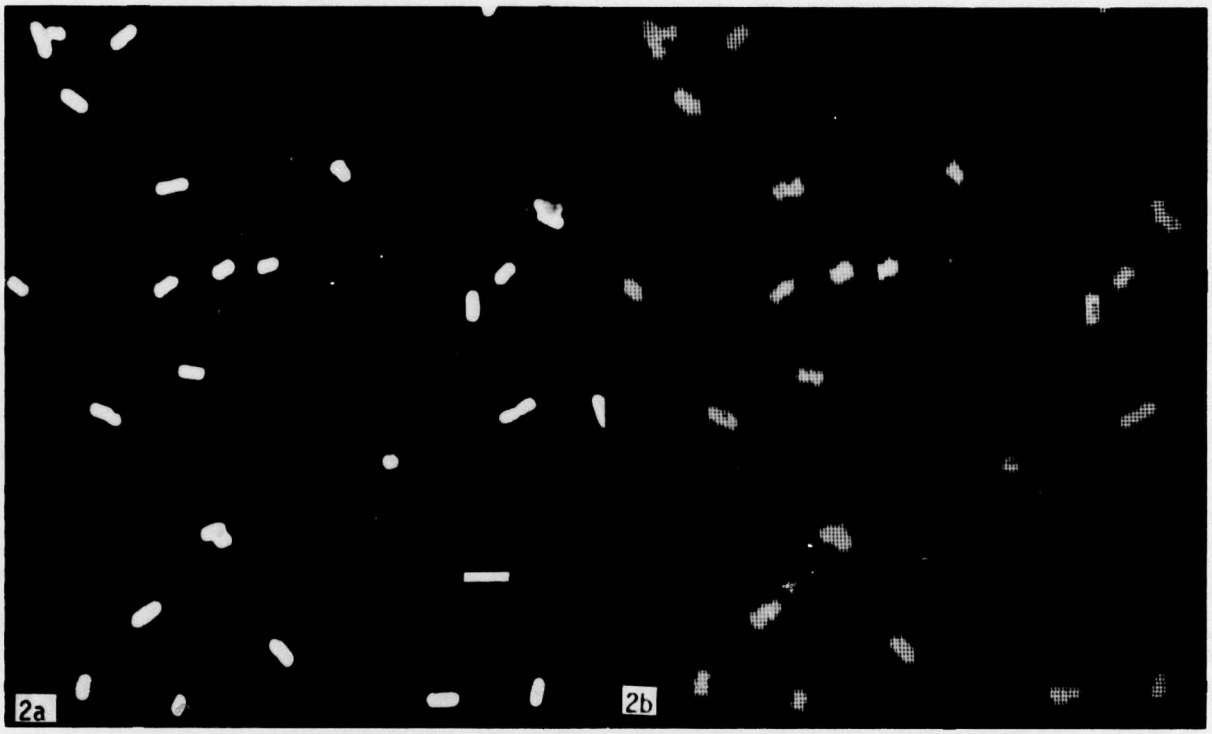
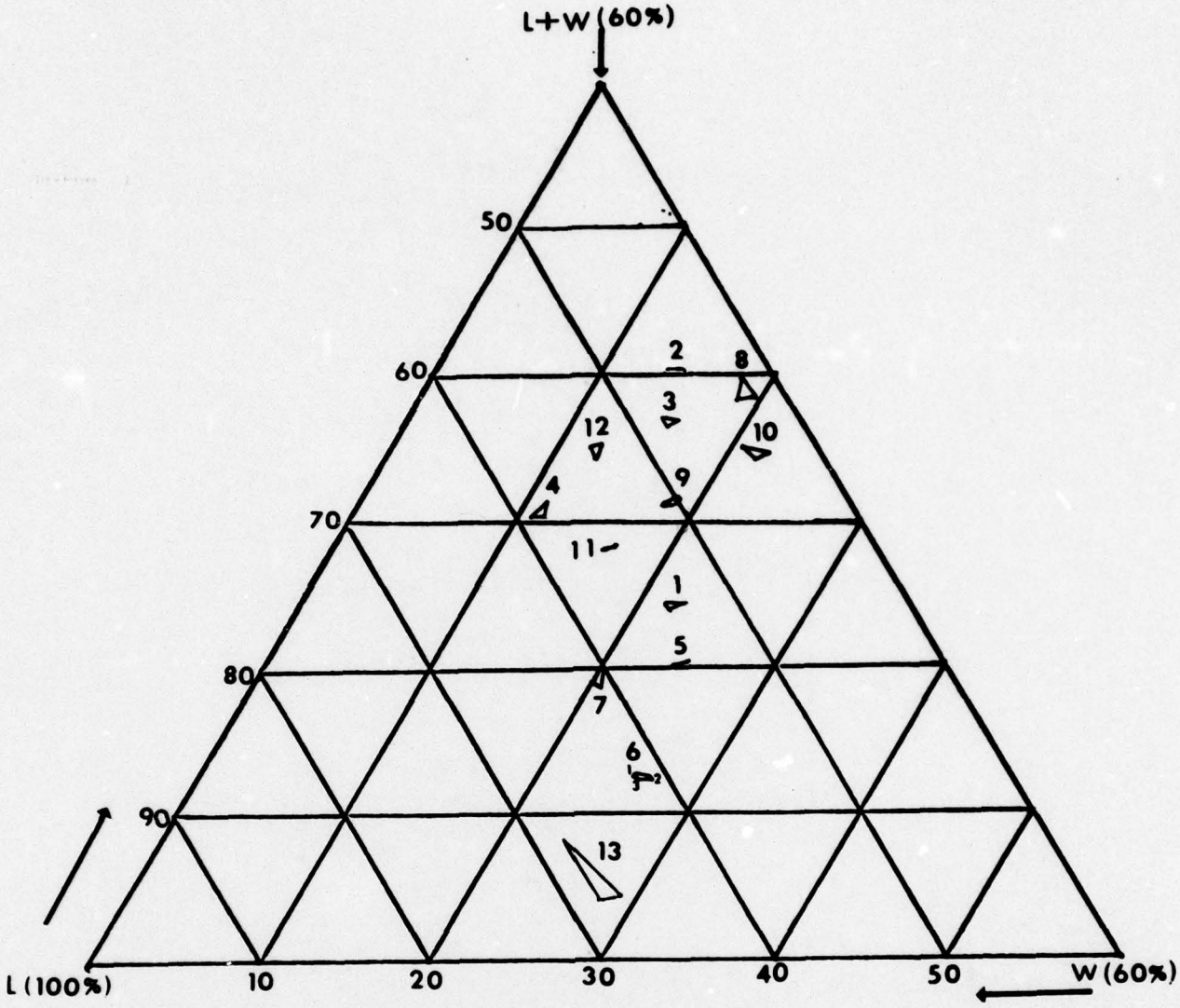


Fig. 2a. Secondary image of Shigella sonnei. Fig. 2b. Demonstrates the binary image of Shigella sonnei (lines in binary image due to electron noise which was filtered out by the computer). Bar length = 2um



1. Enterobacter Aerogenes
2. Enterobacter Agglomerans
3. Enterobacter Cloaca
4. Aeromonas Hydrophila
5. Erwinia Herbicola
6. Escherichia Coli
7. Klebsiella Rhinoscleromatis
8. Proteus Rettgeri
9. Salmolella Enteritidis
10. Serratia Liquefaciens
11. Shigella Sonnei
12. Vibrio Parahaemolyticas
13. Yersinia Enterocolitica

$R_L = 0 - 2.5u$

$R_W = 0 - 2.5u$

$R_{L+W} = 0 - 2.5u$

$\%L = \frac{L}{R_L}$

$\%W = \frac{W}{R_W}$

$\%L+W = 1 - \frac{L+W}{R_{L+W}}$

Figure 3. Ternary diagram for morphometric classification of 13 enteric bacteria.

Appendix I

Construction of the Ternary Diagram

To determine data points on the ternary diagram (Figure 3), particle-by-particle computer data were compared with micrographs of the bacteria measured. Occasionally within a field of "on points" (e.g., a bacterium in the field), electronic noise created an "off point" by reduction of the voltage at that point. This noise at times resulted in premature shortening (by the computer) of chords which are used for width and length determinations. When these shortened chords were far less than the bacteria size range actually observed in micrographs, the data were eliminated from the computer readout and further processing.

Once noise was determined and eliminated from the data, histograms of the frequency distribution of lengths and widths were made. For example, Figure 4 provides the distributions for three runs on E. Coli. From these histograms, geometrical averages were determined and used in the formulas beneath the ternary diagram. Average length, width and length plus width are represented by one point for each run.

It is anticipated that the effect of noise can be virtually eliminated through further improvements in software.

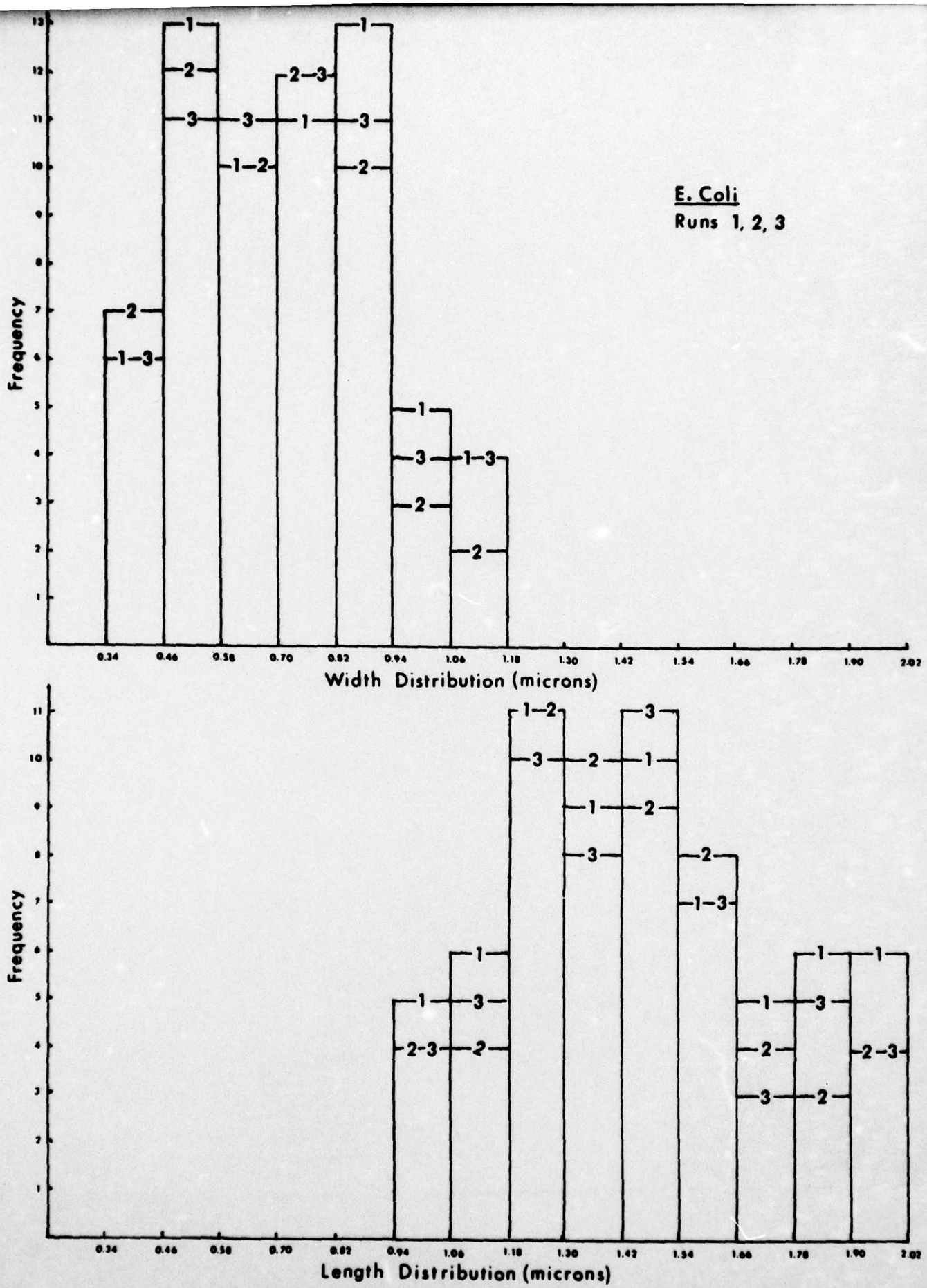


Figure 4. Width and lenath histograms for E. Coli.